FEBS Letters 529 (2002) 356–360

FEBS 26607

Transfection of a phosphatidyl-4-phosphate 5-kinase gene into rat atrial myocytes removes inhibition of GIRK current by endothelin and α-adrenergic agonists

Kirsten Bender, Marie-Cécile Wellner-Kienitz, Lutz Pott*

Department of Physiology, Ruhr-University Bochum, D-4480 Bochum, Germany

Received 18 July 2002; revised 5 September 2002; accepted 5 September 2002

First published online 20 September 2002

Edited by Jacques Hanoune

Abstract GIRK (G protein-activated inward-rectifying K^+ channel) channels, important regulators of membrane excitability in the heart and in the central nervous, are activated by interaction with βγ subunits from heterotrimeric G proteins upon receptor stimulation. For activation interaction of the channel with phosphatidylinositol 4,5-bisphosphate ($PtIns(4,5)P_2$) is conditional. Previous studies have provided evidence that in myocytes $PtIns(4,5)P_2$ levels relevant to GIRK channel regulation are under regulatory control of receptors activating phospholipase C. In the present study a phosphatidyl-4-phosphate 5-kinase was expressed in atrial myocytes by transient transfection. This did not affect basal properties of GIRK current activated by acetylcholine via M_2 receptors but completely abolished inhibition of guanosine triphosphate-γ-*S* activated current by endothelin-1 or α-adrenergic agonists. We conclude that though $PtIns(4,5)P_2$ is conditional for channel gating, its normal level in the membrane is not limiting basal function of GIRK channels. Moreover, our data provide further evidence for a regulation of GIRK channels by α_{1A} receptors and endothelin-A receptors, endogenously expressed in atrial myocytes, via depletion of $PtIns(4,5)P_2$.

Key words: Cardiac myocyte; G protein-activated inward-rectifying K^+ channel; PIP_2; Adrenergic receptor; Endothelin; Muscarinic receptor

1. Introduction

G protein-activated inwardly rectifying K^+ (GIRK) channels expressed in cardiac supraventricular tissue and various types of neurons and endocrine cells couple to many different seven-helix receptors and are important regulators of membrane excitability. Activation of appropriate receptors causes release of βγ subunits from heterotrimeric G proteins. Direct interaction of $G_{\beta\gamma}$ with the subunits of GIRK channels increases their open probability [1,2]. The four cloned mammalian GIRK subunits (GIRK1–4 or Kir3.1–3.4) belong to the family of inwardly rectifying K^+ channels (Kir). Members of this family are either constitutively active or are regulated by a variety of intracellular factors. For some members it has been demonstrated that their gating requires phosphatidylinositol phosphates. Apart from $G_{\beta\gamma}$-mediated activation, which is conditional for transmitter-mediated GIRK current, these channels have been shown to be modulated by phosphatidylinositol 4,5-bisphosphate ($PtIns(4,5)P_2$) and intracellular Na^+ ions [3–5]. Based on these detailed studies analyzing the effects of $PtIns(4,5)P_2$ it is assumed that its binding to the channel is conditional for gating, and that $G_{\beta\gamma}$ stabilizes the channel-$PtIns(4,5)P_2$ interactions.

Recent studies have demonstrated that stimulation of receptors in atrial myocytes, which activate phospholipase C (PLC) via $G_{q/11}$, such as the adrenergic α_1 receptor (α1-AR) or the endothelin-A receptor (ET_A-R) causes inhibition of atrial GIRK current. This inhibition showed clear symptoms of being mediated by depletion of $PtIns(4,5)P_2$ [6–8], supporting the notion that $PtIns(4,5)P_2$ levels required for channel gating are under regulatory control.

The level of $PtIns(4,5)P_2$ in the cell membrane is regulated in a complex manner by a network of PI-kinases and PI-phosphatases and PLC [9–11]. In the classic pathway phosphatidylinositol is phosphorylated to $PtIns(4,5)P_2$ by two successive reactions catalyzed by PI4-kinases and phosphatidyl-4-phosphate 5-kinases (PI(4)P5-K). Manipulation of PI-kinase activity by overexpression of a PI(4)P5-K in a heterologous expression system has been demonstrated to affect sensitivity of co-expressed K_{ATP} channels to ATP, demonstrating the role of this phospholipid for physiological activity of this channel, whose pore-forming subunit (Kir6.x) represents another member of the Kir family [12].

In the present study the α-isoform of a PI(4)P5-K originally cloned from the MIN6 murine pancreatic cell line [13] was expressed in adult rat atrial myocytes. These cells endogenously express functional GIRK1/GIRK4 channels, α_{1a} adrenergic receptors and ET_A receptors, both coupling to $G_{q/11}$, resulting in activation of PLC. This approach was used to study, (i) if the endogenous (basal) level of $PtIns(4,5)P_2$ in the membrane is limiting activation of GIRK channels by M_2 receptors, and (ii) if increasing $PtIns(4,5)P_2$ synthesis affects inhibition of GIRK current via PLC-coupled receptors.

It was found that transfection with the PI(4)P5-K-encoding vector did not affect density of ACh-induced GIRK current or its activation rate respectively. Acute desensitization of whole cell current, which represents a fast and reversible decay of

*Corresponding author. Fax: (49)-234-3214449.
E-mail address: lutz.pott@ruhr-uni-bochum.de (L. Pott).

Abbreviations: GIRK, G protein-activated inward-rectifying K^+ channel; ET, endothelin; $PtIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PI(4)P5-K, phosphatidyl-4-phosphate 5-kinase; PLC, phospholipase C

0014-5793/02/$22.00
PII: S0014-5793(02)03426-9

$I_{K(ACh)}$ observed upon rapid exposure of a cell to a high concentration of ACh or other activating agonists and most likely reflects a property of the channel complex [14] was not affected either. Inhibition of guanosine triphosphate (GTP)-γ-*S* stimulated GIRK current, following activation of the PLC-coupled signalling pathway by exposure to ET-1 or the α-adrenergic agonist phenylephrine respectively, was completely abolished in transfected myocytes. These results support the notion that in atrial myocytes and presumably in other systems, GIRK channels can be regulated by PLC-coupled receptors via depletion of $PtIns(4,5)P_2$. This phospholipid therefore, in the system under study, fulfills the criteria of a second messenger, apart from IP3 and DAG, of the receptor–$G_{q/11}$–PLC pathway. Moreover, without stimulation of PLC-coupled receptors, the endogenous level of $PtIns(4,5)P_2$ does not appear to be limiting normal function of GIRK channels in their native environment.

2. Materials and methods

2.1. Isolation and culture of atrial myocytes

Experiments were performed with local ethics committee approval. Wistar Kyoto rats of either sex (around 200 g) were anaesthetized by i.v. injection of urethane (1 g/kg). The chest was opened and the heart was removed and mounted on the cannula of a sterile Langendorff apparatus for coronary perfusion at constant flow. The method of enzymatic isolation of atrial myocytes and culture conditions have been described in detail elsewhere (e.g. reference [15]). Myocytes were used experimentally from day 0 until day 5 after isolation. No effects of time in culture were found as for the key experiments.

2.2. Solutions and chemicals

For whole cell measurements of atrial GIRK current an extracellular solution of the following composition was used (mmol/l): NaCl 120; KCl 20; $CaCl_2$ 0.5; $MgCl_2$ 1.0; HEPES/NaOH 10.0, pH 7.4. The solution for filling the patch-clamp pipettes for whole cell measurements of G protein-activated K^+ currents contained (mmol/l): K-aspartate 110; KCl 20; NaCl 5.0, $MgCl_2$ 1.0; Na_2ATP 2.0; EGTA 2.0; GTP 0.01; HEPES/KOH 10.0, pH 7.4. Standard chemicals were from Merck (Darmstadt, Germany). EGTA, HEPES, MgATP, GTP, GTP-γ-*S* ACh-chloride, ET-1, phenylephrine, and methoxamine were from Sigma (Deisenhofen, Germany).

2.3. Current measurement

Membrane currents were measured using whole cell patch clamp. Pipettes were fabricated from borosilicate glass and were filled with the solution listed above (DC resistance 4–6 MΩ). Currents were measured by means of a patch-clamp amplifier (WPC-100, ESF, Göttingen, Germany). Signals were analogue filtered (corner frequency of 1–3 kHz), digitally sampled at 5 kHz and stored on a computer, equipped with a hardware/software package (ISO2 by MFK, Frankfurt/Main, Germany) for voltage control and data acquisition. Experiments were performed at ambient temperature (22–24°C). If not otherwise stated, cells were voltage-clamped at −90 mV, i.e. negative to E_K, resulting in inward K^+ currents. Current–voltage relations were determined by means of voltage ramps between −120 mV and +60 mV within 500 ms. Rapid superfusion of the cells for application and withdrawal of agonist-containing solutions was performed by means of a solenoid-operated flow system that permitted switching between up to six different solutions with a half time of exchange ≤100 ms.

2.4. Transfection of myocytes

The cDNA encoding for murine type I PI(4)P5-K α-isoform [16] (kindly provided Dr. Y. Oka, Yamaguchi, Japan) was subcloned into the pAdTrack-CMV vector using *Sal*I and *Xba*I sites (pAd-PI(4)P5-K). The pAdTrack-CMV vector was kindly provided by Dr. B. Vogelstein (Howard Hughes Medical Institute, Baltimore, MD, USA). This vector encodes for GFP as reporter for transfection-positive cells. Transfection was performed using Lipofectamine Plus reagent, according to the manufacturers instructions. Electrophysiological recordings were made on days 3 and 4 after transfection. Transfected cells (< 5%) were identified by epifluorescence of GFP (excitation wavelength 470 nm).

2.5. Statistics

Data are presented as mean ± S.D. and were analyzed using Student's *t*-test for unpaired samples. A value of $P < 0.05$ was considered to be significant.

3. Results

We first investigated if expression of PI(4)P5-K affects basic properties of GIRK current activated by stimulation of

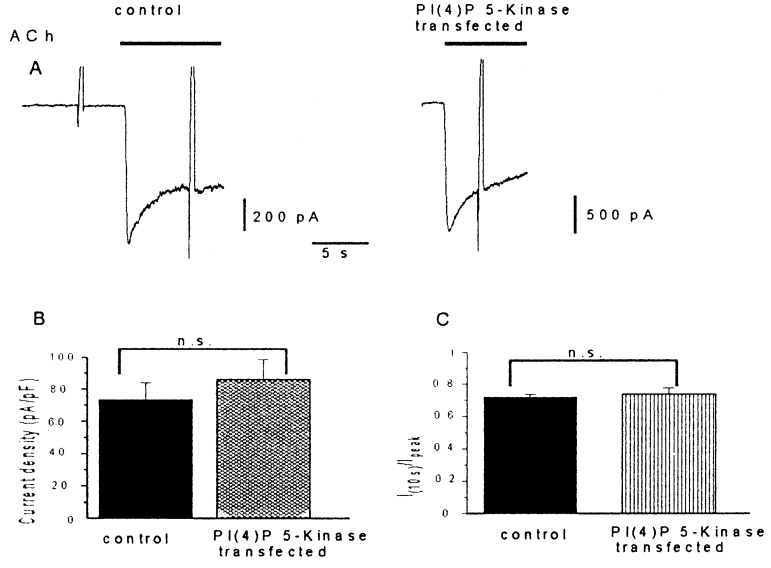

Fig. 1. Transfection with PI(4)P5-K vector does not affect ACh-activated current (20 μmol/l). A: Representative sample recordings from a myocyte transfected with the empty pAdTrack vector (left) and pAd-PI(4)P5-K (right). B: Summarized data on current densities. C: Summarized data on acute desensitization; to qualitatively assess acute (channel) desensitization without contamination by receptor desensitization [38], ratios of current at 10 s after starting superfusion with ACh-containing solution divided by peak inward current were determined. Differences between controls and transfected cells were not significant. ($n = 8$ for each group).

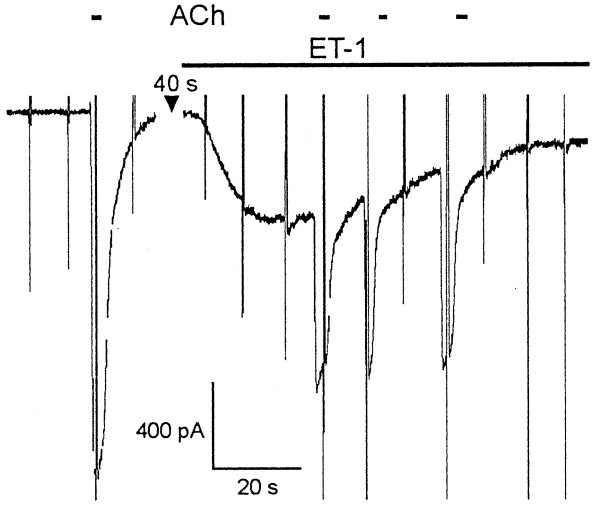

Fig. 2. Simultaneous activation and inhibition of GIRK current by ET-1. ACh (20 μmol/l) and ET-1 (10 nmol/l) were applied as indicated by the horizontal bars. The arrowhead indicates a gap in the recording of 40 s.

M_2AChR. Representative current recordings shown in Fig. 1A and summarized data on current densities (B), and acute desensitization (C) reveal no differences in these parameters between control cells and myocytes transfected with the empty pAdTrack-CMV vector. Corresponding results were obtained, when the $G_{i/o}$-coupled pathway was stimulated by adenosine via A_1 receptors (not shown). This is in line with a previous study demonstrating that inclusion of PtIns(4,5)P_2 in the pipette filling solution does not affect these properties of GIRK current in atrial myocytes [7] and lends supports to the notion that the endogenous level of PIP_2 is not a limiting factor for normal (basal) function of atrial GIRK channels and their activation by $G_{i/o}$-coupled receptors.

Exposure of atrial myocytes to ET_1, in line with previous studies [7,17,18] results in slow activation of GIRK current, most likely reflecting some promiscuous coupling of the ET_A receptor to $G_{i/o}$ and inhibition of the current activated by ACh via stimulation of M_2AChR as illustrated in Fig. 2. The superposition of activation and inhibition shows a large variability between individual cells. Moreover upon activation of $I_{K(ACh)}$ by ET-1 some heterologous desensitization of the response to ACh occurs [7] that cannot be distinguished from a genuine inhibition. Comparable effects, i.e. variable degrees of activation and inhibition were found when α_1 receptors were stimulated by phenylephrine or methoxamine respectively (not shown, see reference [7]).

We therefore used an experimental protocol that allows studying the inhibitory component without contamination by activation or acute desensitization. For this purpose GTP-γ-*S* (500 μmol/l) was routinely included in the pipette filling solution. After a period of ~2 min following rupture of the membrane, one or a series of exposures to ACh resulted in irreversible activation of $I_{K(ACh)}$, which apart from a slow 'rundown' in the order of 20% within ~5 min, remained fairly stable. Upon exposure of a GTP-γ-*S*-loaded myocyte to Phe (100 μmol/l) under this condition, the current decayed with a half time in the order of magnitude of 1 min, in line with our previous study. Fig. 3A,B shows representative traces demonstrating the effect of Phe on GTP-γ-*S*-activated GIRK current in a control myocyte (transfected with the empty vector) and a pAd-PI(4)P5-K transfected cell. The inhibition caused by α_1 receptor stimulation on average amounted to about 70% in myocytes transfected with the empty vector or non-tranfected cells respectively. The inhibition was significantly reduced to 20%, i.e. to the baseline rundown level in the group of transfection-positive myocytes (Fig. 3B). Comparable results were obtained, when ET_1 (10 nM/l) was used as PLC-activating agonist (Fig. 3C,D). The summarized data in Fig. 4 reveal that GIRK current inhibition caused by activation of ET_A-R or α_{1a}-R respectively was reduced to baseline in cells overexpressing the kinase.

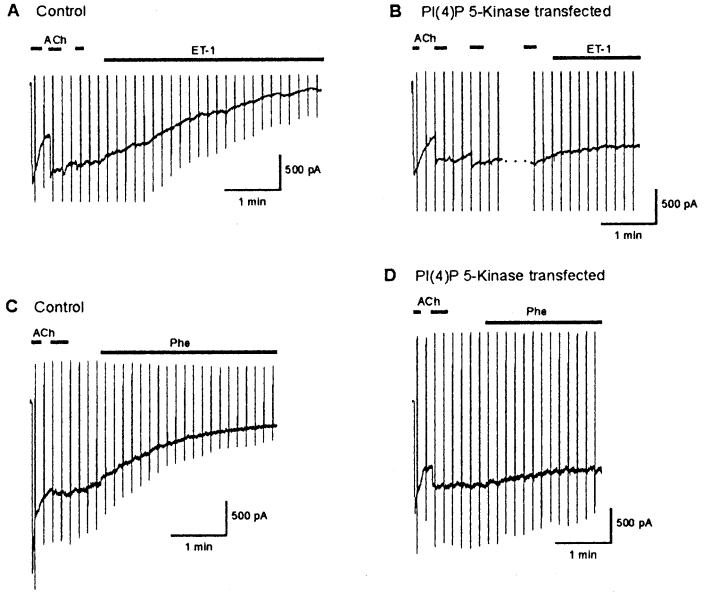

Fig. 3. Sample recordings demonstrating effects of ET-1 (A,B) and phenylephrine (C,D) on GTP-γ-*S* activated GIRK current. In this series of experiments GTP-γ-*S* was included in the pipette filling solution (500 μmol/l). Cells were repetitively challenged by ACh (20 μmol/l) until a stable level of current activation was reached. ACh and ET-1 or Phe were applied as indicated by the horizontal bars.

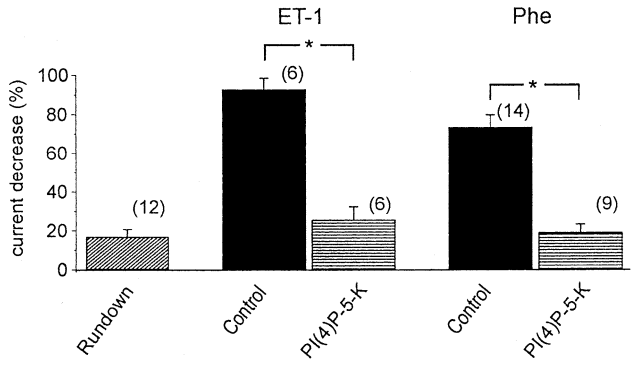

Fig. 4. Summarized data from experiments illustrated in Fig. 3. The bar labelled 'rundown' represents the spontaneous decrease in current 4–5 min after the last challenge by ACh ($n=22$; compare Fig. 3) recorded from cells transfected with the empty vector. Inhibition of GIRK current by ET-1 (10 nmol/l) or Phe (100 μmol/l) was determined in a quasi steady state, i.e. after 3–5 min of exposure to the agonist.

These data clearly demonstrate that increasing an enzyme activity, which contributes to the synthesis of PtIns(4,5)P_2 counteracts the inhibition of an ion channel whose dependence on PtIns(4,5)P_2 has been demonstrated previously.

4. Discussion

GIRK channels are activated by direct interaction with βγ subunits released from heterotrimeric G proteins preferentially but not exclusively of the pertussis toxin-sensitive class ($G_{i/o}$) upon agonist stimulation of appropriate receptor species [19,20]. Apart from this canonical activating pathway other regulatory factors have been identified, such as intracellular Na^+, which appears to be closely linked to regulation by PtIns(4,5)P_2 [21]. Phosphorylation by PKA has been suggested to exert a facilitatory effect GIRK currents in atrial cells [22], which is, however contradictory to studies from other laboratories [23–26]. A complex regulatory pathway modulating voltage-dependent relaxation of GIRK current has been delineated recently [27,28]. According to these studies, there is a tonic inhibition of $G_{\beta\gamma}$-induced channel activity by RGS4 that is reduced by phosphatidylinositol-3,4,5,-trisphosphate (PIP_3). This regulation becomes voltage dependent, since Ca^{2+} entry, via formation of Ca^{2+}/calmodulin, reverses the action of PIP3.

Phosphoinositide metabolism plays an essential role in numerous cellular processes, and controlled synthesis and enzymatic breakdown of phosphoinosites are fundamental signalling events. Sensitivity to PtIns(4,5)P_2 is a property that is shared with other members of the family of inwardly rectifying K^+ (Kir.x) channels, such as ATP-sensitive K^+ channels composed of Kir6.x pore-forming α-subunits and SUR β-subunits [29–32] or the renal ROMK1 channel [33]. However, also channels belonging to other families have been shown to be sensitive to PtIns(4,5)P_2 (e.g. [34,35]). The mechanism(s) of how PtIns(4,5)P_2 affects channel gating are not identical in different channels or other membrane proteins. In $K_{(ATP)}$ channels increasing PtIns(4,5)P_2 results in a decreased apparent affinity for ATP, the physiological inhibitor of channel gating [12]. Evidence has been provided that this reflects a competition between ATP and PtIns(4,5)P_2 for a C-terminal binding site on the Kir6.x subunit [36]. For the GIRK channels it is assumed that binding of PtIns(4,5)P_2 to its subunits is conditional for gating, and that $G_{\beta\gamma}$ stabilizes the interactions between the channel subunit and the phosphoinositide. Although GIRK channel gating strongly depends on the presence of PtIns(4,5)P_2 [21,37] suggesting a regulatory role, it is not clear if basal function of this channel in its native environment is limited by the concentration of this phosphoinositide. The lack of an effect of overexpressing PI(4)P5-K on ACh-induced current under basal conditions clearly suggests that PtIns(4,5)P_2 is not a limiting component for basal function. However, stimulation of endogenous receptors that result in breakdown of PtIns(4,5)P_2 results in inhibition of GIRK channel activity, providing a crosstalk between $G_{i/o}$- and $G_{q/11}$-coupled pathways. At present little is known about regulation of PI(4)P5-K activities by receptor-mediated signals. Such a regulation might provide an additional modulatory input to this system.

Acknowledgements: We thank Anke Galhoff, Bing Liu and Gabiele Reimus for expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Po212/9-3).

References

[1] Dascal, N. (2001) Trends Endocrinol. Metab. 12, 391–398.
[2] Mark, M.D. and Herlitze, S. (2000) Eur. J. Biochem. 267, 5830–5836.
[3] Huang, C.L., Feng, S. and Hilgemann, D.W. (1998) Nature 391, 803–806.
[4] Zhang, H., He, C., Yan, X., Mirshahi, T. and Logothetis, D.E. (1999) Nat. Cell Biol. 1, 183–188.
[5] Ho, I.H. and Murrell-Lagnado, R.D. (1999) J. Physiol. 520, 645–651.
[6] Lei, Q., Talley, E.M. and Bayliss, D.A. (2001) J. Biol. Chem. 276, 16720–16730.
[7] Meyer, T., Wellner-Kienitz, M.-C., Biewald, A., Bender, K., Eickel, A. and Pott, L. (2001) J. Biol. Chem. 276, 5650–5658.
[8] Cho, H., Nam, G.-B., Lee, S.H., Earm, Y.E. and Ho, W.-K. (2001) J. Biol. Chem. 276, 159–164.
[9] Toker, A. (1998) Curr. Opin. Cell Biol. 10, 254–261.
[10] Anderson, R.A., Boronenkov, I.V., Doughman, S.D., Kunz, J. and Loijens, J.C. (1999) J. Biol. Chem. 274, 9907–9910.
[11] Majerus, P.W., Kisseleva, M.V. and Norris, F.A. (1999) J. Biol. Chem. 274, 10669–10672.
[12] Shyng, S.-L., Barbieri, A., Gumusboga, A., Cukras, C., Pike, L., Davis, J.N., Stahl, P.D. and Nichols, C.G. (2000) Proc. Natl. Acad. Sci. USA 97, 937–941.
[13] Ishihara, H., Shibasaki, Y., Kizuki, N., Katagiri, H., Yazaki, Y., Asano, T. and Oka, Y. (1996) J. Biol. Chem. 271, 23611–23614.
[14] Bender, K., Wellner-Kienitz, M.-C., Inanobe, A., Meyer, T., Kurachi, Y. and Pott, L. (2001) J. Biol. Chem. 276, 28873–28880.
[15] Bünemann, M., Brandts, B. and Pott, L. (1996) J. Physiol. (Lond.) 492, 351–362.
[16] Ishihara, H., Shibasaki, Y., Kizuki, N., Katagiri, H., Yazaki, Y., Asano, T. and Oka, Y. (1996) J. Biol. Chem. 271, 23611–23614.
[17] Kim, D. (1991) Circ. Res. 69, 250–255.
[18] Yamaguchi, H., Sakamoto, N., Watanabe, Y., Saito, T., Masuda, Y. and Nakaya, H. (1997) Am. J. Physiol. Heart Circ. Physiol. 273, H1745–H1753.
[19] Dascal, N. (2001) Trends Endocrinol. Metab. 12, 391–398.
[20] Wellner-Kienitz, M.C., Bender, K. and Pott, L. (2001) J. Biol. Chem. 276, 37347–37354.
[21] Ho, I.H.M. and Murrell-Lagnado, R.D. (1999) J. Biol. Chem. 274, 8639–8648.
[22] Mullner, C., Vorobiov, D., Bera, A.K., Uezono, Y., Yakubovich, D., Frohnwieser-Steinecker, B., Dascal, N. and Schreibmayer, W. (2000) J. Gen. Physiol. 115, 547–558.
[23] Wang, Y.G. and Lipsius, S.L. (1995) Circ. Res. 77, 565–574.
[24] Sorota, S., Rybina, R., Yamamoto, A. and Du, X.-Y. (1999) J. Physiol. (Lond.) 514, 413–423.

[25] Wellner-Kienitz, M.-C., Bender, K., Brandts, B., Meyer, T. and Pott, L. (1999) FEBS Lett. 451, 279–283.
[26] Wellner-Kienitz, M.C., Bender, K. and Pott, L. (2001) J. Biol. Chem. 276, 37347–37354.
[27] Ishii, M., Inanobe, A., Fujita, S., Makino, Y., Hosoya, Y. and Kurachi, Y. (2001) Circ. Res. 89, 1045–1050.
[28] Ishii, M., Inanobe, A. and Kurachi, Y. (2002) Proc. Natl. Acad. Sci. USA 99, 4325–4330.
[29] Hilgemann, D.W. and Ball, R. (1996) Science 273, 956–959.
[30] Baukrowitz, T., Schulte, U., Oliver, D., Herlitze, S., Krauter, T., Tucker, S.J., Ruppersberg, J.P. and Fakler, B. (1998) Science 282, 1141–1144.
[31] Shyng, S.L., Cukras, C.A., Harwood, J. and Nichols, C.G. (2000) J. Gen. Physiol. 116, 599–608.
[32] Krauter, T., Ruppersberg, J.P. and Baukrowitz, T. (2001) Mol. Pharmacol. 59, 1086–1093.
[33] Liou, H.H., Zhou, S.S. and Huang, C.L. (1999) Proc. Natl. Acad. Sci. USA 96, 5820–5825.
[34] Bian, J., Cui, J. and McDonald, T.V. (2001) Circ. Res. 89, 1168–1176.
[35] Runnels, L.W., Yue, L. and Clapham, D.E. (2002) Nat. Cell Biol. 4, 329–336.
[36] MacGregor, G.G., Dong, K., Vanoye, C.G., Tang, L., Giebisch, G. and Hebert, S.C. (2002) Proc. Natl. Acad. Sci. USA 99, 2726–2731.
[37] Sui, J.L., Petit-Jacques, J. and Logothetis, D.E. (1998) Proc. Natl. Acad. Sci. USA 95, 1307–1312.
[38] Bünemann, M., Brandts, B. and Pott, L. (1997) J. Physiol. (Lond.) 501, 549–554.